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TITLE: A Polyamine Oxidizing Enzyme as a Drug to Treat Breast Cancer

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14. ABSTRACT The research is aimed at testing two polyethylene glycolated (PEGylated) forms of bovine serum amine oxidase (SAO) as effective treatments for breast cancer using a mouse model. Hopefully, this approach, or a variation thereof, can be used as a new therapy for breast and other cancers in humans. Currently, a large quantity of pure bovine PAO is in hand, which was obtained from 10 gallons of fresh cow blood. A final purification step has been used to produce large quantities of extremely pure SAO. Attempts to find a cost-effective, practical method for the deglycosylation of SAO were unsuccessful. Using several different reagents, we hope to obtain PEG derivatives of SAO. Before testing as anti-tumor agents, both PEGylated SAO derivatives will be tested for toxicity by using nontumorigenic mice. Once it is established that these agents do not have significant side effects, they will be tested for the ability to slow the growth or shrink the size of breast tumors implanted in test mice. PEGylated SAO should target tumors but have little effect on normal tissue. Once concentrated extracellularly in a tumor, active PEGylated SAO will oxidize acetylated polyamines, which are excreted by tumor cells in large quantities. When the acetylated polyamines are oxidized, cytotoxins are generated.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	6
Conclusion.....	6
References.....	6

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INTRODUCTION

We purpose to target specifically breast tumors with high levels of an enzyme that oxidizes efficiently N^1 -acetyl-spermine and N^1 -acetyl-spermidine. These agents are exported from tumor cells at high levels. Presumably, the toxic oxidation products will be generated locally in sufficient quantities to slow or arrest the growth or kill tumor cells without harming substantially non-cancerous tissues (1, 2). For this work, we have chosen bovine serum amine oxidase (SAO), which has been obtained in large quantities in a very pure form from cow blood (3). The work can be considered as nanotechnological in nature since each molecule of SAO is polyethylene glycol(PEG)-encapsulated. This allows the modified enzyme to target tumors with high specificity; due to its high vascularization and unusual nature of the capillaries surrounding a tumor, an intravenously injected PEGylated enzyme will target specifically malignancies but not normal tissues (1, 2, 4). The PEG-coated enzyme has enhanced stability, is protected from proteolysis, and is not antigenic. These properties afford the PEG-enzyme an increased lifetime, and, hence, an increased circulation time relative to the unmodified form (1, 2). The goal of the research is to inject PEGylated SAO into the blood stream of breast tumor-bearing mice to determine if this treatment is a viable anticancer therapy.

BODY

TASK 1. Prepare enough of two polyethylene glycol(PEG)-derivatives of bovine serum amine oxidase (SAO).

In order to obtain the requisite amount of SAO, we procured 10 gallons of fresh cow blood from a local slaughterhouse. By following a published protocol with little modification, we obtained pure SAO (3). While very pure, the SAO still had low levels of contaminants that could possibly interfere with its PEGylation and/or obscure the outcome of experiments to test the treatment as an anticancer therapy. Hence, more time was required to identify a final step to remove the trace contaminants. We found that chromatography on a Macro Prep Type I Ceramic Hydroxyapatite (Bio-RAD) column work very well for this purpose (5). This method was used to prepare enough enzyme for the initial phase of the work, i.e., to determine the toxicity of two PEG-SAO derivatives in non-tumorigenic mice (**TASK 2**).

Initially, we proposed to deglycosylate SAO before attempting PEGylation of the enzyme. It was thought that this would reduce or eliminate any interference with the animal's serum SAO, or the mimicking of this endogenous enzyme. However, it was deemed to be prohibitively expensive to deglycosylate the required amounts of SAO for our experiments; it would cost tens of thousands of dollar to accomplish this goal. This unsuccessful endeavor required about 2 months of exploratory research. Next, we proceeded with the PEGylation of the fully native bovine SAO with the hope that the large PEG groups would partially or fully mask the attached polysaccharide, thus, minimize interference with or mimicking of the mouse's own SAO.

A small amount of bovine SAO was PEGylated at pH 7.4 with Sunbright® ME-050CS (PEG

MW = 5,000 Da) and Sunbright® ME-200CS (PEG MW = 20,000 Da) (NOS Corp., Tokyo, Japan). In addition, this enzyme was PEGylated at pH 8.0 with mPEG-SC MW 5,000 and mPEG-SC MW 20,000 (Laysan Bio Inc., Arab, AL). It was found that the Sunbright® reagents gave good PEGylation levels while retaining high enzymic activity. Next, we initiated work to prepare larger amounts of two PEG-SAO derivatives using these reagents that are needed for **TASK 2**.

When we scaled up the production of the PEGylated derivatives, the level of derivatization was much lower than that resulting from the trial experiments, and too low to be acceptable. A second attempt provided the same results. A high degree of PEGylation is essential because the enzyme is glycosylated, and the protein-bound PEG groups are required to block the polysaccharides. The polysaccharides are required for SAO to act a vascular adhesion protein-1 (VAP-1) (6). If the polysaccharides are not block on SAO, when injected into the blood of test mice, it may interfere with the endogenous VAP-1. In addition, a low-level PEGylation probably will not lower the antigenicity of SAO in the blood of the test animals. Hence, the immune response against the SAO antigen will not allow it to target cancer cells, and defeat the purpose of this study.

In order to obtain the proper PEGylated derivatives, we will try other PEG reagents that will be obtained from Laysan Bio Inc. However, we are unable to predict how much extra time will be required to overcome this unforeseen setback. We believe that, minimally, it will require several months. Hence, we hope, at least, that we will be able to test the toxicity of the PEG derivatives (Task 2), but are uncertain whether enough time and funds will remain after **TASK 2** is completed to work on **TASK 3**.

TASK 2. Test the general toxicity of the two PEG-SAO derivatives.

This work will require injecting several non-tumorigenic mice with one PEG-SAO derivative, and injecting another group of mice with the second derivative. We are unable to predict when this work will be initiated (see above, under **Task 1**). The time required to procure the proper PEG derivative may not leave enough time or funds to accomplish this

TASK 3. Test each PEG-SAO conjugate as an antitumor agent using mice with implanted human tumors.

This task will not be initiated until we prepare a large amount of the two PEGylated forms of bovine SAO (**TASK 1**), and we have tested these in nontumorigenic mice (**TASK 2**).

KEY RESEARCH ACCOMPLISHMENTS

- Procured a large quantity of extremely pure bovine PAO for deglycosylation and PEGylation.
- Attempted to efficiently and cost-effectively deglycosylate bovine SAO. After about 6 weeks of work, this endeavor was deemed untenable.
- Developing methods to produce two PEGylated derivatives of bovine SAO; one form of SAO will have about five 5,000 MW PEG groups attached, and the other form of SAO about five 20,000 MW PEG groups attached.
- Work is in progress to scale-up the production of these two derivatives.

REPORTABLE OUTCOMES

The only reportable outcome is that we have obtained the requisite amount of pure bovine SAO for the remainder of our research on this project, and that we are attempting to develop methods to PEGylated the enzyme.

CONCLUSION

Since we have not yet done any animal work, we cannot report any conclusions. If our hypothesis is correct, the treatment may one day be an effective anticancer therapy in human patients.

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